

# $\alpha$ -Adrenergic response and myofilament activity in mouse hearts lacking PKC phosphorylation sites on cardiac TnI

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**Montgomery, David E., Beata M. Wolska, W. Glen Pyle, Brian B. Roman, Jasmine C. Dowell, Peter M. Buttrick, Alan P. Koretsky, Pedro Del Nido, and R. John Solaro.**  $\alpha$ -Adrenergic response and myofilament activity in mouse hearts lacking PKC phosphorylation sites on cardiac TnI. *Am J Physiol Heart Circ Physiol* 282: H2397–H2405, 2002; 10.1152/ajpheart.00714.2001.—Protein kinase C (PKC)-mediated phosphorylation of cardiac myofilament (MF) proteins has been shown to depress the actomyosin interaction and may be important during heart failure. Biochemical studies indicate that phosphorylation of Ser<sup>43</sup> and Ser<sup>45</sup> of cardiac troponin I (cTnI) plays a substantial role in the PKC-mediated depression. We studied intact and detergent-extracted papillary muscles from nontransgenic (NTG) and transgenic (TG) mouse hearts that express a mutant cTnI (Ser43Ala, Ser45Ala) that lacks specific PKC-dependent phosphorylation sites. Treatment of NTG papillary muscles with phenylephrine (PE) resulted in a transient increase and a subsequent 62% reduction in peak twitch force. TG muscles showed no transient increase and only a 45% reduction in force. There was a similar difference in maximum tension between NTG and TG fiber bundles that had been treated with a phorbol ester and had received subsequent detergent extraction. Although levels of cTnI phosphorylation correlated with these differences, the TG fibers also demonstrated a decrease in phosphorylation of cardiac troponin T. The PKC-specific inhibitor chelerythrine inhibited these responses. Our data provide evidence that specific PKC-mediated phosphorylation of Ser<sup>43</sup> and Ser<sup>45</sup> of cTnI plays an important role in regulating force development in the intact myocardium.

protein kinase C; troponin I

PHOSPHORYLATION OF MYOFILAMENT (MF) proteins may be significant in the transition from compensatory hypertrophy to decompensated heart failure. Our hypothesis (3, 23) has been that activation of protein kinase C (PKC) in response to stressors that induce hypertrophy may not only activate transcription but may also alter

MF activation. In this scenario, maladaptive growth is combined with depressed force development in a viscous cycle leading to end-stage heart failure. Cardiac MFs have multiple sites that are substrates for PKC including myosin light chain 2 (15) and cardiac troponins I (cTnI) and T (cTnT) (17). However, it appears that phosphorylation of cTnI may be especially important in the hypertrophy/failure process. Heart samples from failed myocardium demonstrate an increase in phosphorylation of cTnI (1, 22). Moreover, in vitro determination of the ATPase rate of reconstituted preparations (14) indicated that the PKC-mediated phosphorylation of Ser<sup>43</sup> and Ser<sup>45</sup> on cTnI is particularly important in the depression of the actin-myosin interaction. Yet whether phosphorylation at these sites specifically affects tension generated by the native MF lattice has not been determined. It is also unclear how the effect of phosphorylation of cTnI at Ser<sup>43</sup> and Ser<sup>45</sup> may influence or be influenced by the state of phosphorylation of cTnT, which is one of its neighbors on the thin filament. We recently demonstrated (12) that the phosphorylation state of TnT is also an important determinant of the effect of PKC-dependent phosphorylation on MF tension.

In the experiments reported here, we employed a transgenic (TG) mouse model with hearts expressing a mutant cTnI (Ser43Ala, Ser45Ala) that lacks functionally significant sites for PKC-specific phosphorylation. To test whether these sites are important determinants of the effects of PKC, we compared twitch dynamics of intact papillary muscle preparations from control and TG hearts treated with phenylephrine (PE) in the presence of propranolol. We also measured the Ca<sup>2+</sup>-tension relation of skinned-fiber bundles that had been treated with the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA). The effects of PKC-mediated phosphorylation induced by PE and TPA were significantly reduced in TG preparations com-

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pared with controls. Our data provide the first demonstration that phosphorylation of Ser<sup>43</sup> and Ser<sup>45</sup> on cTnI modulates force generation by the intact myocardium and support the hypothesis that these sites may significantly contribute to regulation of cardiac function in physiological and pathophysiological conditions.

## METHODS

**Materials.** Calyculin A was obtained from Calbiochem. PE, propranolol, okadaic acid, chelerythrine, and TPA were obtained from Sigma.

**TG mice.** The cardiac-specific expression of the mutated cTnI cDNA was driven by a mouse  $\alpha$ -myosin heavy-chain promoter in an FVB/N background as previously described (10). Nontransgenic (NTG) littermates or age- and sex-matched FVB/N mice (Charles River) served as controls. Based on levels of mutant and wild-type mRNA as well as relative levels of protein phosphorylation, we estimate that ~50% of the native cTnI was replaced with mutant TnI. Immunoblot analysis indicated that the total TnI was the same in TG and NTG MFs.

**Force development in intact isolated papillary muscle.** Mice were anesthetized with an injection of 2,2,2-tribromoethanol (125 mg/kg body wt ip). Hearts were quickly removed and perfused with a modified Krebs-Henseleit solution with the following composition (in mM): 118.5 NaCl, 15.0 KCl, 1.2 MgSO<sub>4</sub>, 2.0 NaH<sub>2</sub>PO<sub>4</sub>, 26 NaHCO<sub>3</sub>, 10.0 D-glucose, and 0.4 CaCl<sub>2</sub>. Right ventricular papillary muscles were excised with the tricuspid valves intact. The muscle was mounted in an experimental chamber and perfused with Krebs-Henseleit solution (5 mM KCl), which was equilibrated by bubbling with a 95% O<sub>2</sub>-5% CO<sub>2</sub> gas mixture. Temperature was kept constant at 25.0  $\pm$  0.1°C by use of a heat exchanger at the inflow line and a circulating water bath. The muscles were stimulated at 0.2 Hz via platinum electrodes at a stimulus strength that was 50% above threshold. After equilibration, the Ca<sup>2+</sup> concentration was gradually increased to 1 mM. The muscle was stretched with a servo-controlled motor (Cambridge Technology) to generate 90% of maximum developed force. Propranolol (1  $\mu$ M) superperfusion for 15 min preceded PE (30  $\mu$ M) superperfusion. In some experiments, the muscles were superperfused with chelerythrine (2  $\mu$ M) for 15 min after propranolol and before and during PE perfusion.

**Steady-state tension measurements.** Steady-state tension was measured in detergent-extracted fiber bundles that were dissected from left ventricular papillary muscle and then treated with the phorbol ester TPA as previously described (12). We dissected fiber bundles (~150–200  $\mu$ m wide and 3–4 mm long) and mounted them between a micromanipulator and a force transducer using cellulose-acetate glue. The fibers were immersed (while being stirred) in a chamber of high-relaxing (HR) solution that contained (in mM) 20 MOPS (pH 7.0), 10 EGTA, 1 free Mg<sup>2+</sup>, 5 Mg ATP<sup>2-</sup>, 12 creatine phosphate, and 0.5 dithiothreitol, and 10 U/ml of creatine kinase (bovine heart; Sigma). The initial sarcomere length was set at 2.3  $\mu$ m as determined by laser diffraction, and cross-sectional dimensions were measured. The incubation solution was immediately changed to HR solution containing a cocktail of phosphatase inhibitors (0.1  $\mu$ g/ml of calyculin A and 0.2  $\mu$ g/ml of okadaic acid) and either 100 nM TPA or an equal volume of vehicle, DMSO (control). Treatment with TPA continued for 10 min. In some experiments, fiber bundles were treated with the inactive phorbol ester 4 $\alpha$ -phorbol (100 nM) for 10 min, and in others the bundles were pretreated with the PKC-specific inhibitor chelerythrine (5  $\mu$ M)

for 5 min. After these treatments, fibers were extracted in HR solution that contained 1% Triton X-100 and the phosphatase inhibitors for 30 min. pCa-force relations were then determined as previously described (12). In some experiments, the muscle preparations were incubated with the catalytic subunit of protein kinase A (PKA, 120 U/ml; porcine heart; Sigma) for 45 min under the same buffer conditions as described. The pCa-force measurements were made as described by de Tombe and Stienen (4).

**Labeling of MF proteins with [ $\gamma$ -<sup>32</sup>P]ATP.** To determine phosphate incorporation into MF proteins, we incubated NTG and TG fiber bundles in HR solution that contained 0.1 mM cold ATP and 75  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP as previously described (12).

**Polyacrylamide gel electrophoresis and autoradiography.** SDS polyacrylamide (12.5%) analytic gels were run on the same day as the treatments as described previously (12). Phosphorylation of TnI and TnT in treatment groups from the same day was expressed as a percent increase in <sup>32</sup>P incorporation with vehicle (DMSO) treatment taken as 100%.

**Subcellular fractionation and Western blot analysis.** Fractionation of ventricular muscle was modified from the method of Huang et al. (8). To test the fractionation of PKC isoforms under the conditions of the steady-state force measurements, ventricular strips were incubated with either 100 nM TPA or an equal volume of DMSO in HR solution for 10 min. To test the fractionation of PKC isoforms under the conditions of the intact papillary force measurements, intact ventricular strips were incubated with 30  $\mu$ M PE in Krebs-Henseleit solution for 10 min. Immediately after this, 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and 0.01 mM leupeptin were added to the samples, which were then briefly homogenized (Polytron). Samples were subsequently sonicated for 5 min to solubilize the proteins. Differential centrifugation to obtain the MF, membrane, and cytosolic fractions was as previously described (8). Protein content was measured, and the fractions were completely solubilized in 1% SDS as previously described (12). An equal volume of gel-loading buffer was added, and samples were equally loaded (40  $\mu$ g/lane) onto 8% SDS polyacrylamide gels. Gels were transferred to nitrocellulose membranes using a semi-dry apparatus (Bio-Rad). Western blots were blocked with 10% nonfat dry milk in PBS (0.1% Tween 20) and were subsequently probed using the anti-PKC- $\epsilon$  and anti-PKC- $\alpha$  monoclonal antibodies via horseradish peroxidase-conjugated goat anti-mouse IgG (Jackson Laboratories). Bound monoclonal antibody was detected using the enhanced chemiluminescence (ECL) detection assay (Amersham).

**Statistical analysis.** Data from the normalized pCa-tension relations were fitted to the Hill equation as previously described (2) by using a nonlinear least-square regression procedure to obtain the pCa<sub>50</sub> (negative log of the free Ca<sup>2+</sup> concentration required for half-maximum activation). Statistical differences were analyzed by unpaired Student's *t*-test or one-way ANOVA with Student-Newman-Keuls post hoc analysis for multiple comparisons with significance set at *P* < 0.05. We used one-way ANOVA with repeated measures and subsequent comparison to the least significant difference to analyze differences when means were compared with 100%. All data were expressed as means  $\pm$  SE.

## RESULTS

**Effects of PE on intact papillary muscles.** Treatment of mouse papillary muscles with 30  $\mu$ M PE in the presence of propranolol resulted in a biphasic response. In NTG muscles (Fig. 1A), the initial response (*phase i*)

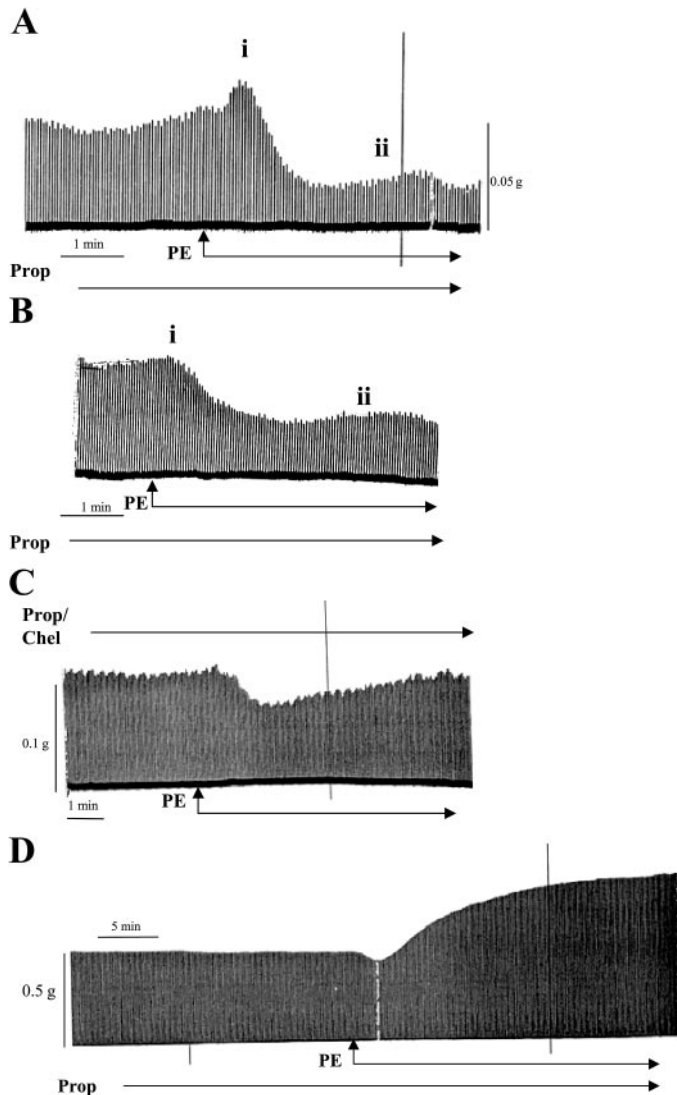


Fig. 1. Comparison of inotropic effects of phenylephrine (PE) in papillary muscles from nontransgenic (NTG) and transgenic (TG; cardiac troponin I-Ser43,45Ala) mouse hearts. **A**: effect of 30  $\mu$ M PE on developed force in control NTG papillary muscles. Basal force was taken as the steady developed force in the presence of 1  $\mu$ M propranolol (Prop). Response to PE was biphasic with an initial (*phase i*) transient positive inotropic effect ( $147 \pm 8.9\%$  of basal developed force in the NTG) followed by a steady (*phase ii*) depression in developed force. **B**: effect of 30  $\mu$ M PE on developed force in TG papillary muscles. Compared with NTG controls, *phase i* was absent in TG preparations and *phase ii* was more depressed. **C**: effect of pretreatment with 2  $\mu$ M chelerythrine (Chel) before 30  $\mu$ M PE treatment. **D**: effect of 30  $\mu$ M PE on force generated by rat papillary muscles.

was a transient increase in the developed force ( $147 \pm 8.9\%$  of basal level) followed by a reduced steady developed force (*phase ii*) compared to the basal developed force. In the TG myocardium (Fig. 1B), PE treatment did not induce a transient phase but did result in a decreased steady developed force (*phase ii*). Figure 1C shows that 2  $\mu$ M chelerythrine preperfusion for 15 min inhibited the transient phase (*phase i*) in response to PE. In addition, the steady phase (*phase ii*) was depressed to a much smaller extent than in the prep-

arations without chelerythrine. These data indicate that both phases were due to PKC activation. We also tested rat papillary muscles under the same conditions to determine whether the negative inotropic response was peculiar to our experimental protocol. As shown in Fig. 1D, in contrast to the case with mouse heart preparations, PE treatment of rat papillary muscles resulted in a significant, steady increase in developed force. These data agree with the findings of Sabri et al. (21), who reported distinct differences in the signaling pathways that lead to PKC activation between the mouse and rat.

Figure 2 shows examples of developed force and twitch dynamics of NTG (Fig. 2A) and TG (Fig. 2B) papillary muscles in the presence of propranolol (basal) and after the PE-induced steady phase (*phase i*). In NTG muscles, there was a 62% decrease in the devel-

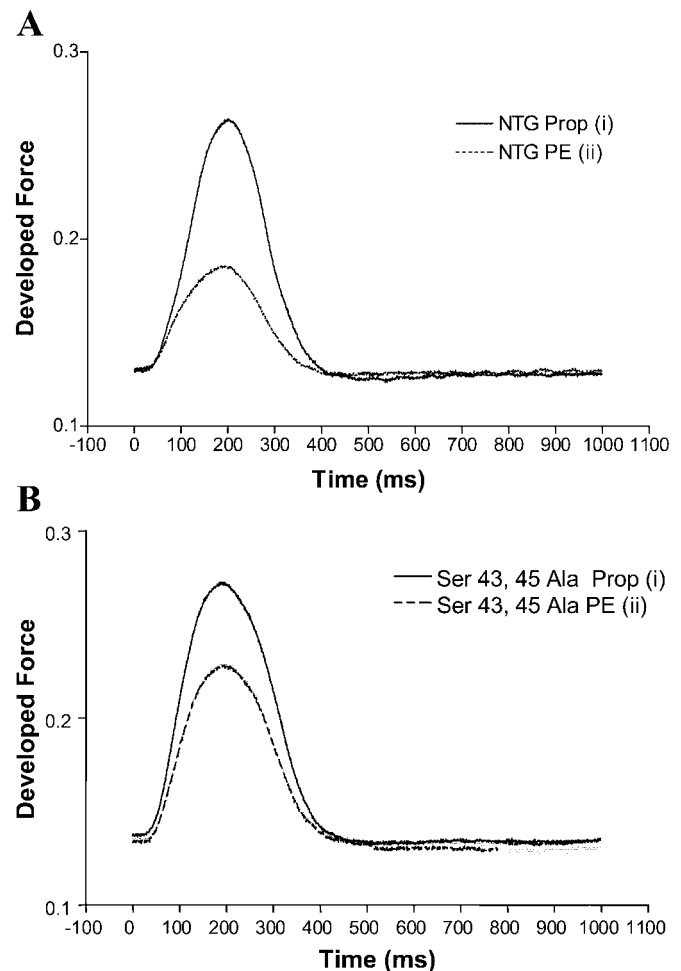


Fig. 2. Comparison of developed force (g) and twitch dynamics of papillary muscles from NTG vs. TG hearts. Representative twitch of NTG (**A**) and TG (**B**) papillary muscles before (solid line) and after (dotted line) PE treatment. Basal force in the presence of 1  $\mu$ M Prop was taken as 100%. PE-induced depression in developed force during the steady phase was  $38.6 \pm 4.7\%$  of basal tension for NTG ( $n = 6$ ) and  $55.3 \pm 3.9\%$  of basal tension for TG ( $n = 6$ ) muscles. Dynamics of contraction and relaxation were not significantly different before and after PE treatment in either NTG ( $n = 6$ ) or TG ( $n = 6$ ) preparations. There were also no significant differences in contraction or relaxation dynamics found between NTG and TG muscles.



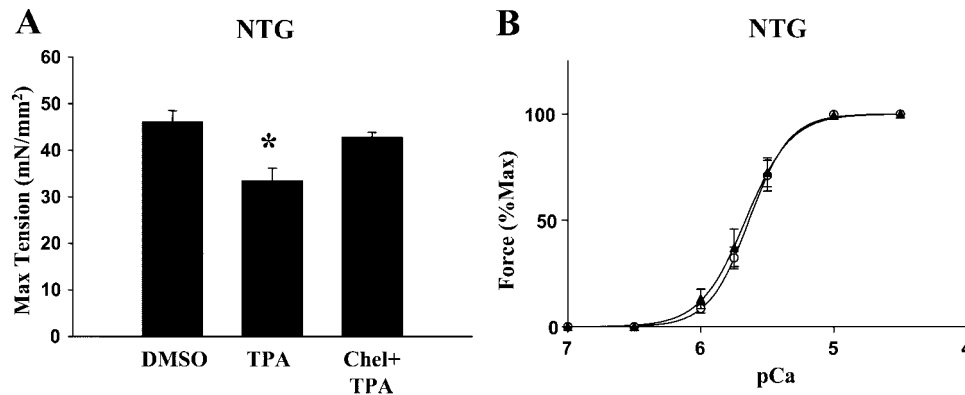


Fig. 3. Effect of protein kinase C (PKC) on  $\text{Ca}^{2+}$ -dependent tension of skinned-fiber bundles from NTG mice. Fiber bundles were detergent extracted after 10-min treatment with the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA, 100 nM) in the presence of a cocktail of phosphatase inhibitors (see METHODS). **A**: comparison of the maximum steady-state tension generated in DMSO-treated controls and TPA-treated preparations in the presence and absence of Chel. TPA decreased tension by 30%, and 5  $\mu\text{M}$  Chel pretreatment for 5 min blocked the decrease in tension. **B**: comparison of pCa-tension relations for DMSO-treated (○) and TPA-treated (▲) fiber bundles. Data are presented as means  $\pm$  SE;  $n = 6$  for DMSO- and TPA-treated groups;  $n = 3$  for Chel-treated group; \* $P \leq 0.01$ , significantly different from DMSO-treated controls.

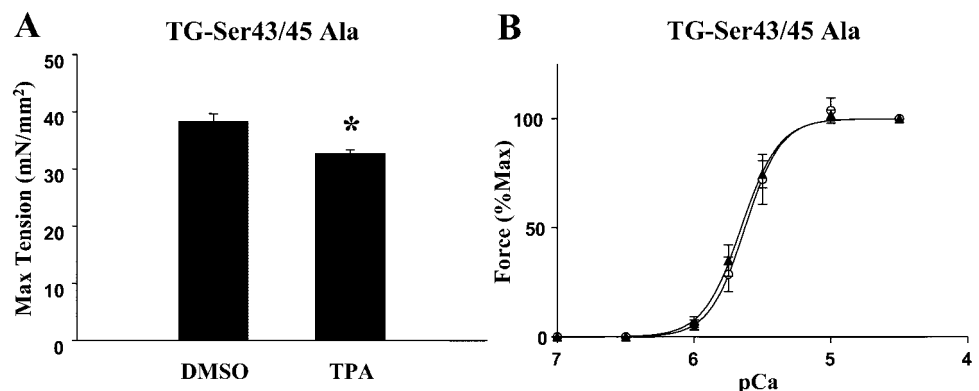
oped tension ( $38.6 \pm 4.7\%$  of basal force) after PE treatment in *phase ii*, whereas in the TG muscles, PE induced only a 45% decrease ( $55.4 \pm 3.9\%$  of basal force). PE did not alter the time to peak force or the time to 75% or 90% relaxation in either NTG or TG muscles. Moreover, when compared with one another, the NTG and TG muscles demonstrated statistically identical twitch dynamics before and after PE treatment (data not shown).

**Effects of TPA on MF activation in detergent-extracted fiber bundles.** The results from intact muscles demonstrated that the absence of PKC-specific sites on cTnI alters the outcome of PKC activation. To test whether these sites contribute to PKC-mediated modifications in MF activity, we measured the pCa-tension relation in NTG and TG fiber bundles that had been treated with TPA and had subsequently been detergent skinned. Figure 3A illustrates that treatment of NTG cardiac fiber bundles with 100 nM TPA in the presence of phosphatase inhibitors (0.1  $\mu\text{g}/\text{ml}$  of calyculin A, 0.2  $\mu\text{g}/\text{ml}$  of okadaic acid) for 10 min decreased the maximum developed tension by 30% ( $45.5 \pm 2.5$  mN/mm<sup>2</sup> for controls vs.  $32.3 \pm 2.7$  mN/mm<sup>2</sup> for TPA-treated preparations). Phosphatase inhibitors alone or

an inactive phorbol ester did not significantly affect tension or  $\text{pCa}_{50}$  (data not shown). To test whether the TPA-induced effect on maximum tension was due to PKC-mediated phosphorylation, we pretreated NTG fiber bundles with the specific PKC inhibitor chelerythrine (5  $\mu\text{M}$ ) for 5 min before adding TPA. Figure 3A shows that the inhibitor abolished the TPA-induced decrease in maximum isometric tension development. There was no effect of TPA treatment on the  $\text{Ca}^{2+}$  sensitivity in NTG MFs as measured by  $\text{pCa}_{50}$  of the normalized pCa-tension relation. The  $\text{pCa}_{50}$  values were  $5.66 \pm 0.01$  in the control group and  $5.63 \pm 0.01$  in the TPA-treated group (Fig. 3B).

In TG fiber bundles, which lacked phosphorylatable Ser residues on cTnI at positions 43 and 45, the maximum tension in the control DMSO-treated TG preparations was 20% lower than the DMSO-treated NTG preparations (Fig. 4A). This result agrees with the data of Noland et al. (14), who previously reported that apart from PKC-dependent phosphorylation, substitution of Ser<sup>43</sup> and Ser<sup>45</sup> with Ala depressed maximum ATPase activity of heavy meromyosin reacting with reconstituted thin filaments. TPA treatment depressed the maximum tension significantly ( $P < 0.001$ ) by 15%

Fig. 4. Effect of PKC on  $\text{Ca}^{2+}$ -dependent tension of skinned-fiber bundles from TG mice. Fiber bundles were detergent extracted after 10-min treatment with 100 nM TPA in the presence of phosphatase inhibitors (see METHODS). **A**: comparison of the maximum steady-state tension generated in DMSO-treated controls and in TPA-treated preparations, in which tension was decreased by 15%. **B**: comparison of pCa-tension relations for DMSO-treated (○) and TPA-treated (▲) fiber bundles. Data are presented as means  $\pm$  SE;  $n = 6$  for DMSO- and TPA-treated groups; \* $P \leq 0.001$ , significantly different from DMSO-treated controls.



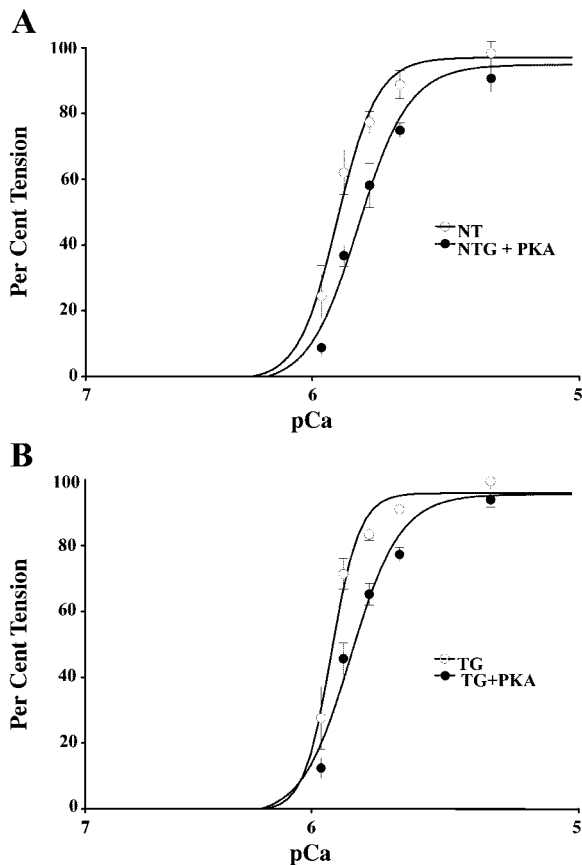


Fig. 5. Effect of protein kinase A (PKA) on  $\text{Ca}^{2+}$ -dependent tension of skinned-fiber bundles from NTG and TG mice. Detergent-extracted fiber bundles were treated for 45 min with PKA, and the  $\text{Ca}^{2+}$ -dependent tension was measured according to de Tombe and Stienen (4). A: PKA treatment of NTG fiber bundles resulted in the well-characterized decrease in  $\text{Ca}^{2+}$  sensitivity (NTG control  $\text{EC}_{50}$ ,  $1.33 \pm 0.01$  vs. PKA  $\text{EC}_{50}$ ,  $1.55 \pm 0.06$   $\mu\text{M}$   $\text{Ca}^{2+}$ ) compared with control. B: PKA treatment of TG fiber bundles resulted in a similar decrease in  $\text{Ca}^{2+}$  sensitivity (TG control  $\text{EC}_{50}$ ,  $1.39 \pm 0.10$  vs. PKA  $\text{EC}_{50}$ ,  $1.82 \pm 0.03$   $\mu\text{M}$   $\text{Ca}^{2+}$ ) compared with control. PKA treatment did not result in a statistically significant difference between NTG and TG preparations nor did PKA alter maximum tension development. For both groups,  $n = 5$  from 3 separate hearts.

( $38.3 \pm 1.2$  for controls vs.  $32.7 \pm 0.5$   $\text{mN}/\text{mm}^2$  for TPA-treated group) in TG preparations (Fig. 4A). The inhibition of maximum tension in NTG fiber bundles was significantly greater than in the TG fibers. As shown in Fig. 4B, the  $\text{Ca}^{2+}$  sensitivity was unaltered by TPA (control  $\text{pCa}_{50}$ ,  $5.65 \pm 0.01$  vs. TPA-treated  $\text{pCa}_{50}$ ,  $5.62 \pm 0.01$ ) and was not different compared with NTG controls (Fig. 4B).

It was important to confirm that the changes in maximum tension generated in the TG model in response to PKC activation were due to the absence of phosphorylatable amino acids specific for PKC and were not merely a result of the transgenesis. We used PKA-dependent phosphorylation under the same conditions as a control, because the PKA-specific sites had not been changed. Figure 5A shows that PKA-mediated phosphorylation in NTG preparations results in a significant decrease in the  $\text{Ca}^{2+}$  sensitivity (NTG control  $\text{EC}_{50}$ ,  $1.33 \pm 0.01$  vs. PKA  $\text{EC}_{50}$ ,  $1.55 \pm 0.06$   $\mu\text{M}$

$\text{Ca}^{2+}$ ) without changing the tension generated at maximal  $\text{Ca}^{2+}$  concentrations. As shown in Fig. 5B, the well-characterized decrease in  $\text{Ca}^{2+}$  sensitivity (TG control  $\text{EC}_{50}$ ,  $1.39 \pm 0.10$  vs. PKA  $\text{EC}_{50}$ ,  $1.82 \pm 0.03$   $\mu\text{M}$   $\text{Ca}^{2+}$ ) is also seen with the TG preparations in response to PKA-mediated phosphorylation. There were no significant differences in the  $\text{Ca}^{2+}$  sensitivity between NTG and TG after PKA treatment nor was the maximum tension development altered by PKA.

**Effects of PKC activation on MF phosphorylation.** Figure 6A shows the results of experiments aimed at identification of proteins phosphorylated under the conditions of our experiments. Autoradiography (Fig. 6A) demonstrated that only TnI and TnT were phosphorylated under our experimental conditions. As shown in Fig. 6B, TPA treatment caused an increase in  $^{32}\text{P}$  incorporation into TnT (150% of control) and into cTnI (220% of control). Pretreatment of fiber bundles

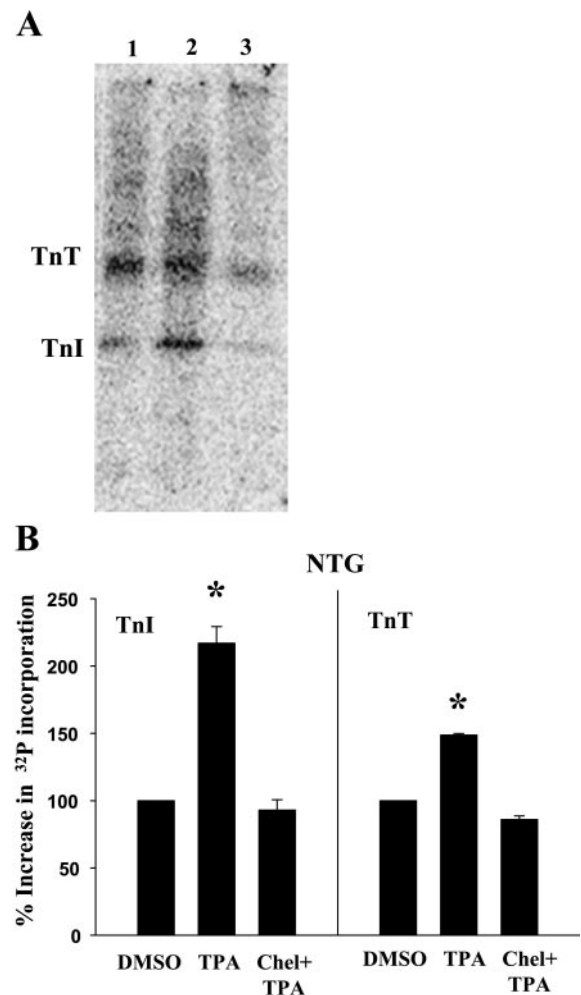


Fig. 6. PKC-mediated  $^{32}\text{P}$  incorporation into NTG myofilaments (MFs). A: lanes 1–3, autoradiogram of MFs in the presence of DMSO (lane 1), TPA (lane 2), or Chel and subsequent TPA treatment (lane 3). B: summary of the quantification of  $^{32}\text{P}$  incorporation after background correction. DMSO control was taken as 100%. Experiments were done in the presence of phosphatase inhibitors. Data are presented as means  $\pm$  SE for 3 separate experiments. \* $P < 0.05$ , significantly different from DMSO control.

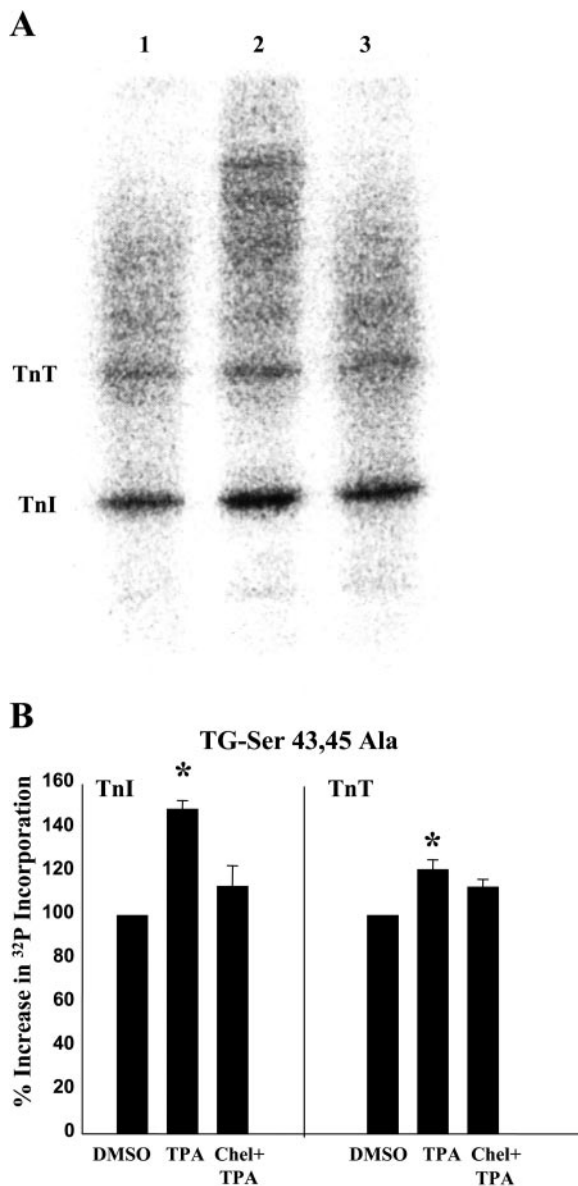


Fig. 7. PKC-mediated  $^{32}\text{P}$  incorporation into TG MFs that lack Ser<sup>43</sup> and Ser<sup>45</sup>. A: lanes 1–3, autoradiogram of MFs in the presence of DMSO (lane 1), TPA (lane 2), or Chel and subsequent TPA treatment (lane 3). B: summary of the quantification of  $^{32}\text{P}$  incorporation after background correction. DMSO control was taken as 100%. Experiments were done in the presence of phosphatase inhibitors. Data are presented as means  $\pm$  SE for 3 separate experiments. \* $P < 0.05$ , significantly different from DMSO control.

with the specific PKC inhibitor chelerythrine (5  $\mu\text{M}$ ) decreased the TPA-induced  $^{32}\text{P}$  incorporation to control levels in cTnT and cTnI (Fig. 6A, lane 3, and Fig. 6B). The phosphorylation profile of TG MFs in Fig. 7A indicated that  $^{32}\text{P}$  incorporation into both TnI and TnT was significantly less than that in the NTG MF preparations. Data summarized in Fig. 7B show that TPA treatment of TG MFs caused a 48% increase in  $^{32}\text{P}$  incorporation into TnI and a 24% increase into cTnT.

**PKC isoform translocation.** It is well appreciated that phorbol esters activate multiple diacylglycerol-sensitive isoforms of PKC and that other, more physi-

ological means of PKC activation (i.e.,  $\alpha$ -adrenergic receptor stimulation) may not result in the same pattern of isoform distribution and thus result in a different functional outcome (26). We used Western analysis to determine the differences in PKC isoform translocation under our experimental conditions. Figure 8A shows the effects of the phorbol ester TPA on translocation of PKC- $\epsilon$  and PKC- $\alpha$  under the same conditions described for the detergent-extracted fiber-bundle experiments (see METHODS). TPA led to translocation of PKC- $\epsilon$  from the cytosolic and membrane fractions to the MF fraction. In contrast, TPA did not lead to translocation of PKC- $\alpha$  to the MF fraction. The small change in the membrane fraction of PKC- $\alpha$  was accounted for by the small increase in the cytosolic fraction. Owing to the low signal, the membrane fractions in Fig. 8A were exposed for a longer time to enhance the signal (Fig. 8A). The effects of PE on PKC-isoform

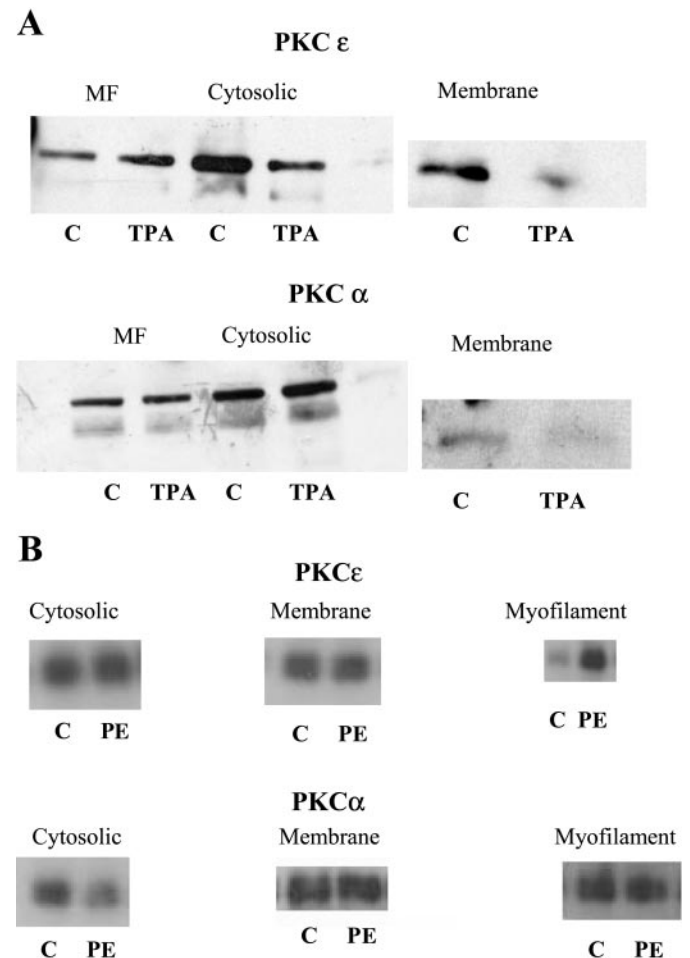


Fig. 8. Western blot demonstrating TPA- and PE-induced translocation of PKC isoforms. A: blots show that the phorbol ester TPA induced PKC- $\epsilon$  translocation to the MF fraction, whereas it did not alter PKC- $\alpha$  distribution compared with DMSO-treated control (C). MF and cytosolic fractions were exposed for 1 min, whereas the membrane fractions were exposed for 3–4 min to optimize signal detection. B: blots show that PE induced translocation of PKC- $\epsilon$  to MF but not PKC- $\alpha$  compared with controls. These blots were exposed for 0.3–2 min. In all experiments, each lane was loaded with 40  $\mu\text{g}$  of protein.



translocation were tested under the conditions described for the intact papillary muscle experiment (see METHODS). Figure 8B shows that PE treatment led to translocation of PKC- $\epsilon$  to the MF fraction but did not alter the distribution of PKC- $\alpha$ . Therefore, the different mechanisms of PKC activation resulted in a similar pattern of PKC- $\epsilon$  and PKC- $\alpha$  distribution. A similar pattern of PKC isoform redistribution was found in the TG samples (not shown). In contrast to the effect of the phorbol ester, translocation of PKC- $\epsilon$  to the MF fraction in response to PE did not result in total redistribution of the isoform throughout the fractions. Although it is not entirely clear why this occurs, it may be an important difference between phorbol ester-induced- and receptor-mediated activation of PKC.

## DISCUSSION

Our data add new understanding regarding the contractile effects of  $\alpha$ -adrenergic receptor stimulation in mouse papillary muscle and are the first to demonstrate a specific role for Ser<sup>43</sup> and Ser<sup>45</sup> of cTnI as sites of PKC-dependent phosphorylation regulating maximum tension in the intact MF lattice. Moreover, our studies on papillary muscle directly implicate MF phosphorylation as important in the response of the myocardium to  $\alpha$ -adrenergic stimulation. Both direct activation and  $\alpha$ -receptor-mediated activation of the PKC-signaling cascade reduced force significantly more in NTG preparations than in TG preparations that lacked these phosphorylation sites. Our studies support our hypothesis (12) that the overall PKC-mediated effects on the MFs are dependent on the phosphorylation status of both TnI and TnT. Furthermore, these results confirm and extend earlier data derived from reconstituted preparations, which suggest that the presence of Ser<sup>43</sup> and Ser<sup>45</sup> on cTnI significantly influences strong cross-bridge binding to the thin filament (14).

*Murine response to  $\alpha$ -adrenergic stimulation.* On the basis of data presented here together with reports in the literature (6, 21), we conclude that the murine signaling pathway for PKC activation differs from that in other species. We directly compared effects of PE on rat and mouse papillary muscles and demonstrated a positive inotropic effect in the rat and a negative inotropic effect in the mouse. Indeed, other species respond to selective stimulation of the  $\alpha$ -adrenergic receptor pathway with a positive inotropic effect (see Ref. 23 for review). These differences in the functional outcome of  $\alpha$ -adrenergic receptor activation reflect differences that have been documented in signal transduction in neonatal (21) as well as adult (7) heart cells.

The results of our studies indicate that Ser<sup>43</sup> and Ser<sup>45</sup> play a role in the transient positive and steady-state negative inotropic effects of PE on papillary muscles. Support for this comes from the finding that both components of the PE-induced biphasic response in the NTG muscles were blunted in the TG papillary muscles. The initial transient increase in force may be partly due to a PKC-mediated increase in Ca<sup>2+</sup> cur-

rent. Nishimaru et al. (13) showed that the L-type Ca<sup>2+</sup> current increased after PE treatment in mice. This increase in Ca<sup>2+</sup> current paralleled the transient increase in force development. Interestingly, the increased Ca<sup>2+</sup> current extended well into the time when the force development decreased in these muscles. Alternatively, the transient increase could be due to changes in the activity of the Na<sup>+</sup>/H<sup>+</sup> exchanger. PKC-mediated phosphorylation of the Na<sup>+</sup>/H<sup>+</sup> exchanger results in intracellular alkalosis and ultimately to increased MF activation (6, 18, 28). Indeed, both mechanisms could combine to affect the ultimate cellular function. Whatever the cellular basis, it is clear that this transient phase was mediated by PKC, because chelerythrine blocked this effect in our experiment. More importantly, there was no transient increase in force in PE-treated TG muscles that lacked Ser<sup>43</sup> and Ser<sup>45</sup>. The mechanism for the loss of the transient increase in force is not clear. One possible explanation for the lack of the PE-induced transient phase is that the TG preparations operate at a near-maximal contractile state. We have demonstrated (19) that basal contractility of these same TG hearts in closed-chest experiments is essentially unaffected by isoproterenol treatment and already near the maximum that could be obtained with isoproterenol treatment of NTG hearts. Furthermore, we found no changes in MF Ca<sup>2+</sup> sensitivity between the NTG and TG muscles before or after PKC-mediated phosphorylation in skinned preparations. Because of this, we have no reason to think that the response of the TG MFs to changes in intracellular pH would be different than those of NTG MFs.

The negative inotropic effect of PE in mouse papillary muscles could be due to a variety of mechanisms that involve the PKC pathway. We have shown that the absence of Ser<sup>43</sup> and Ser<sup>45</sup> considerably reduces the depressive effect induced by PKC-mediated phosphorylation of the MFs. Therefore, MF phosphorylation plays a significant role in the negative inotropic phase in mouse papillary muscles. There had been some debate as to the role of Ca<sup>2+</sup> during this negative inotropic effect. It now appears that just as in the signaling pathways (7, 21), the PKC substrates (i.e., L-type Ca<sup>2+</sup> channel) and therefore the functional outcome of its activation differ in an unpredictable manner across species. For example, studies with rat (9) and rabbit (5) hearts indicated no PE-induced change in the Ca<sup>2+</sup> transient. However, Nishimaru et al. (13) showed that PE increased the Ca<sup>2+</sup> current in adult mice. What is important to point out is that the Ca<sup>2+</sup> current remained elevated even after the negative inotropic phase of the biphasic force development had begun. This finding does not fit with the role of a decreased Ca<sup>2+</sup> current in the negative inotropic phase in mice. Nishimaru et al. (13) concluded from experiments using a variety of inhibitors that the depression of force after treatment with PE was not due to altered activity of L-type Ca<sup>2+</sup> channels, the ryanodine receptor, the Na<sup>+</sup>/H<sup>+</sup> exchanger, the Na<sup>+</sup>/K<sup>+</sup> pump, K<sup>+</sup> channels, or Na<sup>+</sup> channels. However, inhibition of the Na<sup>+</sup>/Ca<sup>2+</sup> exchanger or superfusion with zero Na<sup>+</sup>

(i.e., no exchanger activity) greatly reversed the negative inotropic effect of PE in the mouse-heart preparations. Thus these experiments strongly suggest that  $\alpha$ -adrenergic stimulation is associated with an increase in efflux of  $\text{Ca}^{2+}$  through the  $\text{Na}^+/\text{Ca}^{2+}$  exchanger. Our results clearly indicate that PKC-induced changes in MF activity also play an important role in determining the inotropic state after  $\alpha$ -adrenergic stimulation of the mouse heart.

**MF activation and the PKC pathway.** It is apparent from our studies of detergent-extracted fiber bundles that phosphorylation of cTnI at Ser<sup>43</sup> and Ser<sup>45</sup> is responsible, at least in part, for a PKC-mediated decrease in tension that ultimately affects contractility. However, there are other MF substrates for PKC that may act in concert with cTnI to affect MF activation. cTnT is an important substrate for PKC and has been shown to decrease MF activity exclusively of TnI (15, 17). In our previous studies (12), we reported that compared with controls, PKC-mediated cTnI phosphorylation is significantly depressed in skinned-fiber bundles from TG mice that express fast skeletal TnT, which naturally lacks PKC sites that are present in cTnT. These data indicated that the state of TnT affects cTnI as a substrate for PKC and that interactions among thin-filament proteins may be an important determinant of the effect of PKC-dependent phosphorylation in regulating tension. Results presented here support our hypothesis that PKC-mediated phosphorylation of cTnI or cTnT in the MF lattice is not mutually exclusive. Ser<sup>43</sup> and Ser<sup>45</sup> of cTnI are located in a critical near-NH<sub>2</sub>-terminal region that forms an interface with troponin C and TnT, and we expected that the present experiments would demonstrate an influence of the state of cTnI on cTnT phosphorylation. In fact, this is what we found from our studies comparing phosphorylation of cTnI and cTnT in TG and NTG preparations. In the NTG skinned-fiber bundles, tension was significantly depressed in association with a 220% increase in phosphorylation of cTnI and a 150% increase in phosphorylation of cTnT. In TG preparations, this effect of PKC activation was significantly blunted in association with a 48% drop in phosphorylation of cTnI lacking Ser<sup>43</sup> and Ser<sup>45</sup>, but there was also a 24% drop in phosphorylation of cTnT. Thus these data reveal the complexities of covalent modifications in this region of the thin filament. Interestingly, the effect of PKA-dependent phosphorylation (at Ser<sup>23</sup> and Ser<sup>24</sup>) was not statistically different between the NTG and TG preparations. Aside from this providing an excellent control for the study, it suggests that in the native MF lattice, the regions of the NH<sub>2</sub> terminus of TnI are functionally discrete and far enough removed from the PKC sites such that even changes in the primary structure of TnI sites that substantially alter TnT phosphorylation do not significantly alter the effect of PKA-mediated phosphorylation (i.e., MF response to  $\beta$ -adrenergic stimulation).

In our study, we found a depression in maximal MF tension with no change in MF  $\text{Ca}^{2+}$  sensitivity after PKC activation in the skinned-fiber bundles. This re-

sult fits well with studies reporting that PKC-mediated phosphorylation of the MFs results in reduced cross-bridge binding to the thin filament and a consequent reduction in maximum actomyosin ATPase rate (24, 26). Yet others have reported alterations in  $\text{Ca}^{2+}$  sensitivity associated with PKC-dependent phosphorylation. This may reflect complex interactions among the sites of PKC-dependent phosphorylation on cTnI and cTnT and phosphorylation of myosin light chain 2 (27) or myosin binding protein C (29), proteins that are shown to alter  $\text{Ca}^{2+}$  sensitivity. Thus we cannot entirely rule out the possibility that phosphorylation of sites other than cTnI and cTnT contributes to the effects of  $\alpha$ -adrenergic agonists in the intact papillary muscles. It is likely that the overall functional outcome is dependent on the net phosphorylation state within the cell, which includes all substrates.

PKC not only acutely alters the contractile state of the heart, but it also regulates more long-term changes in the gene expression (1). Depending on which isoform is expressed and active, the phosphorylation profile may change substantially. The complement of PKC isoforms is altered during the progression to heart failure. Quantitative immunoblotting demonstrated that expression of the  $\text{Ca}^{2+}$ -dependent PKC isoforms PKC- $\alpha$ , PKC- $\beta$ I, and PKC- $\beta$ II was substantially increased in failing human hearts, whereas other isoforms were essentially unchanged (1). Interestingly, PKC- $\beta$  isoforms are virtually absent in adult rat hearts but are expressed in large amounts in the developing hearts of the embryo and fetus (20). Conversely, the density of PKC- $\epsilon$ , the most abundant isoform in the adult cardiomyocyte, doesn't appear to change significantly during development (20). For these reasons, we determined the distribution of PKC- $\epsilon$  compared to PKC- $\alpha$ , a  $\text{Ca}^{2+}$ -dependent isoform. Our results indicate that PKC- $\epsilon$  is responsible for the TPA- and PE-induced alterations in MF activity and contractility.

Although the difference in the negative inotropic effect of PE between NTG and TG preparations strongly supports a role for cTnI phosphorylation at Ser<sup>43</sup> and Ser<sup>45</sup>, cTnI has a third site at Thr<sup>144</sup> that is a substrate for PKC. Thr<sup>144</sup> is located in the otherwise highly conserved inhibitory region and is replaced by a Pro in the skeletal TnI sequence. Together, these points suggest that phosphorylation of Thr<sup>144</sup> may also have functional significance in the heart, yet the functional role of this third putative PKC site remains unclear. Although Noland et al. (17) first reported that phosphorylation of Thr<sup>144</sup> may be functionally significant, subsequent studies (27) using site-directed mutagenesis and reconstituted thin filaments indicated that PKC-mediated phosphorylation of Ser<sup>43</sup> and Ser<sup>45</sup> is largely responsible for the depression of maximum actomyosin ATPase rate. However, employing a mutant protein, Malhotra et al. (11) concluded that Thr<sup>144</sup> in the inhibitory peptide of cTnI is the site responsible for PKC-induced depression in MF  $\text{Ca}^{2+}$  sensitivity. Clearly, more work is needed to understand the role of Thr<sup>144</sup>.

We conclude the following: 1) signaling through  $\alpha$ -adrenergic receptors in mouse heart differs from



other species including the rat, 2) the negative inotropic effect of  $\alpha$ -adrenergic stimulation in mouse heart is due in part to phosphorylation of the MFs, and 3) the depression of MF tension is induced through PKC-mediated phosphorylation of specific sites on cTnI as well as altered protein-protein interactions that influence the state of phosphorylation of cTnT.

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## REFERENCES

- Bowling N, Walsh RA, Song G, Estridge T, Sandusky GE, Fouts RL, Mintze K, Pickard T, Roden R, Bristow MR, Sabbah HN, Mizrahi JL, Gromo G, King GL, and Vlahos CJ. Increased protein kinase C activity and expression of  $\text{Ca}^{2+}$ -sensitive isoforms in the failing human heart. *Circulation* 99: 384–391, 1999.
- Chandra M, Montgomery DE, Kim JJ, and Solaro RJ. The N-terminal region of troponin T is essential for the maximal activation of rat cardiac myofilaments. *J Mol Cell Cardiol* 31: 867–880, 1999.
- De Tombe PP and Solaro RJ. Integration of cardiac myofilament activity and regulation with pathways signaling hypertrophy and failure. *Ann Biomed Eng* 28: 991–1001, 2000.
- De Tombe PP and Stienen GJ. Protein kinase A does not alter economy of force maintenance in skinned rat cardiac trabeculae. *Circ Res* 76: 734–741, 1995.
- Endoh M and Blinks JR. Actions of sympathomimetic amines on the  $\text{Ca}^{2+}$  transients and contractions of rabbit myocardium: reciprocal changes in myofibrillar responsiveness to  $\text{Ca}^{2+}$  mediated through  $\alpha$ - and  $\beta$ -adrenoceptors. *Circ Res* 62: 247–265, 1988.
- Gambassi G, Spurgeon HA, Ziman BD, Lakatta EG, and Capogrossi MC. Opposing effects of  $\alpha_1$ -adrenergic receptor subtypes on  $\text{Ca}^{2+}$  and pH homeostasis in rat cardiac myocytes. *Am J Physiol Heart Circ Physiol* 274: H1152–H1162, 1998.
- Hilal-Dandan R, Kanter JR, and Brunton LL. Characterization of G-protein signaling in ventricular myocytes from the adult mouse heart: differences from the rat. *J Mol Cell Cardiol* 32: 1211–1221, 2000.
- Huang XP, Pi Y, Lokuta AJ, Greaser ML, and Walker JW. Arachidonic acid stimulates protein kinase C- $\epsilon$  redistribution in heart cells. *J Cell Sci* 110: 1625–1634, 1997.
- Litwin SE, Vatner DE, and Morgan JP. Inotropic effects of  $\alpha_1$ -adrenergic agonists in myocardium from rats with postinfarction heart failure. *Am J Physiol Heart Circ Physiol* 269: H1553–H1563, 1995.
- MacGowan GA, Du C, Cowan DB, Stamm C, McGowan FX, Solaro RJ, Koretsky AP, and del Nido PJ. Ischemic dysfunction in transgenic mice expressing troponin I lacking protein kinase C phosphorylation sites. *Am J Physiol Heart Circ Physiol* 280: H835–H843, 2001.
- Malhotra A, Nakouzi A, Bowman J, and Buttrick P. Expression and regulation of mutant forms of cardiac TnI in a reconstituted actomyosin system: role of kinase dependent phosphorylation. *Mol Cell Biochem* 170: 99–107, 1997.
- Montgomery DE, Chandra M, Huang QQ, Jin JP, and Solaro RJ. Transgenic incorporation of skeletal TnT into cardiac myofilaments blunts PKC-mediated depression of force. *Am J Physiol Heart Circ Physiol* 280: H1011–H1018, 2001.
- Nishimaru K, Kobayashi M, Matsuda T, Tanaka Y, Tanaka H, and Shigenobu K.  $\alpha$ -Adrenoceptor stimulation-mediated negative inotropism and enhanced  $\text{Na}^+/\text{Ca}^{2+}$  exchange in mouse ventricle. *Am J Physiol Heart Circ Physiol* 280: H132–H141, 2001.
- Noland TA Jr, Guo X, Raynor RL, Jideama NM, Averyhart-Fullard V, Solaro RJ, and Kuo JF. Cardiac troponin I mutants. Phosphorylation by protein kinases C and A and regulation of  $\text{Ca}^{2+}$ -stimulated MgATPase of reconstituted actomyosin S-1. *J Biol Chem* 270: 25445–25454, 1995.
- Noland TA Jr and Kuo JF. Protein kinase C phosphorylation of cardiac troponin I or troponin T inhibits  $\text{Ca}^{2+}$ -stimulated actomyosin MgATPase activity. *J Biol Chem* 266: 4974–4978, 1991.
- Noland TA Jr and Kuo JF. Phosphorylation of cardiac myosin light chain 2 by protein kinase C and myosin light chain kinase increases  $\text{Ca}^{2+}$ -stimulated actomyosin MgATPase activity. *Biochem Biophys Res Commun* 193: 254–260, 1993.
- Noland TA Jr, Raynor RL, and Kuo JF. Identification of sites phosphorylated in bovine cardiac troponin I and troponin T by protein kinase C and comparative substrate activity of synthetic peptides containing the phosphorylation sites. *J Biol Chem* 264: 20778–20785, 1989.
- Puceat M, Clement-Chomienne O, Terzic A, and Vassort G.  $\alpha_1$ -Adrenoceptor and purinoceptor agonists modulate  $\text{Na}^+/\text{H}^+$  antiporter in single cardiac cells. *Am J Physiol Heart Circ Physiol* 264: H310–H319, 1993.
- Roman B, Montgomery D, Ping P, Geenen DL, Solaro RJ, and Buttrick PM. Targeted mutation of protein kinase C phosphorylation sites on cardiac troponin I alters cardiac function in vivo (Abstract). *Circulation* 102: 160, 2000.
- Rybin VO and Steinberg SF. Protein kinase C isoform expression and regulation in the developing rat heart. *Circ Res* 74: 299–309, 1994.
- Sabri A, Muske G, Zhang H, Pak E, Darrow A, Andrade-Gordon P, and Steinberg SF. Signaling properties and functions of two distinct cardiomyocyte protease-activated receptors. *Circ Res* 86: 1054–1061, 2000.
- Sakata Y, Hoit BD, Liggett SB, Walsh RA, and Dorn GW. Decompensation of pressure-overload hypertrophy in  $\text{G}_{\alpha_q}$ -overexpressing mice. *Circulation* 97: 1488–1495, 1998.
- Solaro RJ. Heart failure and the response of cardiac myofilaments to  $\text{Ca}^{2+}$ . *Heart Failure* 10: 150–155, 1994.
- Strang KT and Moss RL.  $\alpha_1$ -Adrenergic receptor stimulation decreases maximum shortening velocity of skinned single ventricular myocytes from rats. *Circ Res* 77: 114–120, 1995.
- Terzic A, Puceat M, Vassort G, and Vogel SM. Cardiac  $\alpha_1$ -adrenoceptors: an overview. *Pharmacol Rev* 45: 147–175, 1993.
- Venema RC and Kuo JF. Protein kinase C-mediated phosphorylation of troponin I and C-protein in isolated myocardial cells is associated with inhibition of myofibrillar actomyosin MgATPase. *J Biol Chem* 268: 2705–2711, 1993.
- Venema RC, Raynor RL, Noland TA Jr, and Kuo JF. Role of protein kinase C in the phosphorylation of cardiac myosin light chain 2. *Biochem J* 294: 401–406, 1993.
- Vila-Petroff M, Perez GN, Alvarez B, Cingolani HE, and Mattiazzi A. Mechanism of negative lusitropic effect of  $\alpha_1$ -adrenoceptor stimulation in cat papillary muscles. *Am J Physiol Heart Circ Physiol* 270: H701–H709, 1996.
- Winegrad S. Myosin binding protein C, a potential regulator of cardiac contractility. *Circ Res* 86: 6–7, 2000.